



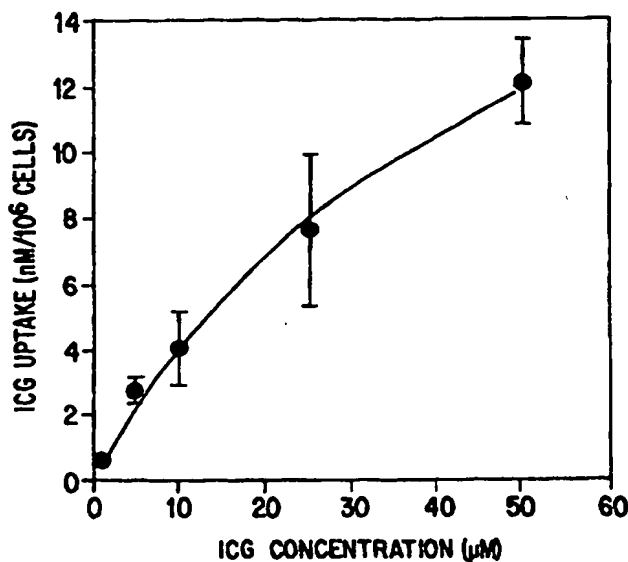
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: A NOVEL PHOTOTHERAPEUTIC METHOD FOR TREATING CANCER AND/OR DERMATOLOGICAL DISEASES AND CONDITIONS

(57) Abstract

The present invention concerns a novel method of treating a cancer, dermatological disease or condition, or combination thereof by administering a physiologically acceptable dye having an absorption maximum of from 770 nm to 840 nm and irradiating with light in the same wavelength range. In preferred embodiments, the dye is indocyanine green, the light source is a diode laser, and the light dose is phototherapeutically effective, as opposed to photothermally effective.



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TITLE OF THE INVENTION

A NOVEL PHOTOTHERAPEUTIC METHOD FOR TREATING CANCER  
AND/OR DERMATOLOGICAL DISEASES AND CONDITIONS

BACKGROUND OF THE INVENTIONField of the Invention:

The present invention concerns a novel method for phototherapeutic treatment of cancer, particularly highly vascularized types, e.g. Kaposi's sarcoma, and of dermatological diseases and conditions, particularly vascular malformations, e.g., hemangiomas, port wine stains, varicosis, etc.

Discussion of the Background:

Indocyanine green (ICG), a dye approved for use in humans by the United States Food and Drug Administration, has been widely applied in medical diagnosis since 1956<sup>1</sup> for measurement of cardiac output<sup>2</sup>, determination of blood volume and plasma volume<sup>3</sup>, hepatic function studies<sup>4</sup>, ophthalmic angiography<sup>5</sup>, capillary microscopy<sup>6</sup>, lung water function and object localization in tissue<sup>7,8</sup>. Indocyanine green exhibits a low incidence of adverse reactions<sup>9,10</sup> and has been characterized physicochemically<sup>11,12,13,14,15</sup> and pharmacokinetically<sup>16,17</sup>. The absorption spectrum of this water-soluble, anionic tricarboyanine dye displays a strong peak between 790 and 810

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nm, coincident with the emission wavelength of a commercially available diode laser (805 nm)). ICG has been used in vivo with near infrared light for dye-enhanced tissue welding<sup>18,19</sup>, treatment of photosclerosis<sup>20</sup>, and induction of photocoagulation<sup>21,22</sup>.

During the past few decades, especially since appropriately powerful lasers and flexible fiber optic light delivery systems became available, an expanding effort has been devoted to the use of lasers in the treatment of cancer and dermatological conditions. Diseases characterized by cellular hyperproliferation and neovascularization are also targets for laser therapy. Alternative and distinct modes of action for laser light are commonly employed<sup>23,24</sup>.

In standard applications, the proliferating cells are destroyed by focused laser light which photothermally destroys the tissue, be it benign or malignant. Coincident use of an appropriate chromophore which localizes in the microvasculature, stroma or cells of the diseased tissue can serve to enhance the destruction of the selected lesions either by photothermal or photodynamic/photochemical (PDT) effects<sup>25</sup> and provide additional selectivity of the treatment to protect surrounding normal tissue.

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ICG is a well-known dye (see The Merck Index, 11th ed., Merck & Co., Rahway, New Jersey (1989), pp. 785-786), which is also known as CardioGreen and Fox Green. The compound is ionic and has a molecular composition  $C_{41}H_{47}N_2NaO_6S_2$ . The chromophore has an adequately broad absorption beyond the peak absorption at 805 nm *in vitro* (due to the binding to plasmaproteins, in particular  $\alpha_1$ -lipoproteins with a molecular weight of approx. 150,000-200,000 Da).

Due to its high molecular weight, ICG does not leave the normal vasculature *in vivo* and is apparently metabolized only in the liver. However, due to insufficient angiogenesis in a variety of diseases (e.g., solid tumors, inflammatory disorders, etc.), these vessels have an increased fragility which renders them more susceptible towards vascular targeting by PDT or photothermolysis. Moreover, due to an increased vascular permeability of the microcirculation in the diseased areas associated with dermatological and/or oncological disorders, ICG is able to leave the microcirculation in the diseased areas and accumulate selectively in dermatological and/or oncological lesions. This has been demonstrated by videomicroscopy on human beings with Kaposi's sarcoma or metastases of colon carcinoma on the skin.

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Recently, the use of ICG has been described for chromophore-enhancement in photothermal destruction of cadaver tumor tissue in vitro (Chen et al, Cancer Lett., vol. 88 (1995), pages 15-19). A diode laser emitting light at 808 nm was employed. In the absence of ICG, the laser inflicted no apparent tissue damage with irradiance up to 1755 J/cm<sup>2</sup>. However, the laser-tissue photothermal interaction in ICG-targeted tissue showed laser damage. For example, the *in vitro* photothermal effects on breast tumor cells at light doses of from 441 to 501 J/cm<sup>2</sup> were characterized by the loss of cytoplasmic elements and by clefts in the tissue field created by the shrinkage of the connective tissue.

Clinical conditions for use of ICG appear to be somewhat critical, as has been well illustrated by Wang and Densmore (see Hepatology, October 1995, page 110A). In their comparative study of ICG with two known photosensitizers (methylene blue and haematoporphyrin derivative), ICG did not appear to be a photosensitizer under the conditions of use.

One aspect of potential success in treatment of cancer and/or dermatological conditions is the physical appearance of the patient. Hair loss and nausea (generally associated with conventional chemotherapy) or scarring (generally associated with surgical techniques and photothermal/photoablation treatment) are

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undesirable, and can cause psychological problems which may hamper or impede the patient's recovery. Thus, a need exists for a phototherapeutic treatment of cancer and dermatological conditions which is unlikely to cause hair loss or nausea and which does not destroy the affected tissues and thus potentially cause scar formation.

#### SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide a novel method of treating cancer which can avoid the adverse effects associated with conventional chemotherapy, surgery and photodestruction.

A further object of the present invention is to provide a novel method of treating cancer which is capable of eliminating cancer cells *in vivo* to a level below the level of clinical detection yet not induce significant scarring, nausea, hair loss or other adverse conditions.

A further object of the present invention is to provide a novel and safe method for treatment of dermatological diseases and conditions which reduces the risks of scarring associated with conventional surgery and laser surgery.

These and other objects of the present invention, which will be readily understood in the context of the following detailed

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description of the preferred embodiments, have been provided by a novel method of treating cancer and/or a dermatological disease or condition, comprising administering to a patient in need of such treatment an effective amount of indocyanine green (which has a light absorption maximum in the range of from 770 to 840 nm), and irradiating the affected or apparently affected tissue of the patient with a dose of light having a wavelength within the range of from 770 to 840 nm, the dose of light being effective to therapeutically treat the cancer and/or dermatological disease or condition, but preferably ineffective to thermally destroy the irradiated tissue.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the intracellular uptake of ICG into HaCaT keratinocytes after 24 hours of incubation with different extracellular ICG concentrations; and

Figure 2 is a graph showing the results of concentration and light dose finding studies based on the cell viabilities of HaCaT keratinocytes treated with ICG (incubation time 24 hours) at different concentrations, irradiated using a cw-diode laser (805 nm; irradiance 40 mW/cm<sup>2</sup>).



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DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the context of the present invention, a "dermatological disease and/or condition" refers to any dermatological and/or cosmetic physiological condition treatable by pharmaceutical therapy or by conventional or laser surgery, including vascular malformations (hemangiomas, port wine stains, varicose veins, telangiectasias), dermatofibromas, keloids disease-induced lesions (e.g., those caused by human papilloma virus [HPV]), cosmetic conditions such as wrinkles, moles, dysplastic nevi, birthmarks, etc. Cancers such as basal and squamous cell carcinomas, malignant melanomas, Paget's disease (either extramammary or of the nipple), Kaposi's sarcoma, etc. may be considered dermatological diseases, cancers or both.

The phrase "thermal destruction" refers to heat-induced cell necrosis. Typically, temperatures necessary for thermal destruction are 50 °C or greater. By contrast, the term "phototherapy" refers to application of relatively low doses of light, such as those which are intended to generate singlet oxygen without substantial heating of the irradiated cell or tissue. For example, a phototherapeutic dose of light typically results in cellular temperatures of less than 50 °C, preferably 45 °C or less, more preferably 43 °C or less and most preferably 41 °C or less.

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For example, light doses which generally are effective for phototherapy but ineffective for photothermal destruction include those of  $250 \text{ J/cm}^2$  or less, preferably  $200 \text{ J/cm}^2$  or less, more preferably  $100 \text{ J/cm}^2$  or less, and even more preferably  $60 \text{ J/cm}^2$  or less. A minimum effective light dose for phototherapy may be at least  $10 \text{ J/cm}^2$ , preferably at least  $25 \text{ J/cm}^2$ , and more preferably at least  $40 \text{ J/cm}^2$ . By contrast, doses greater than  $250 \text{ J/cm}^2$  may be effective for photothermal destruction (see Chen et al, supra).

In addition, laser power densities may have an effect on phototherapeutic effectiveness as compared to photothermal effectiveness. For example, photothermal therapy typically will employ a high power density of, for example,  $10$  to  $20 \text{ W/cm}^2$ . By contrast, the present invention preferably irradiates at a relatively low fluence rate (power density) of less than  $10 \text{ W/cm}^2$ , preferably from  $5 \text{ mW/cm}^2$  to  $5 \text{ W/cm}^2$ , more preferably from  $10 \text{ mW/cm}^2$  to  $3 \text{ W/cm}^2$ , even more preferably from  $25 \text{ mW/cm}^2$  to  $2 \text{ W/cm}^2$ , and most preferably from about  $40$  to  $500 \text{ mW/cm}^2$ . It should be noted, however, that deeper-seated tumors may be more effectively treated with a higher power density of light (e.g.,  $2$ - $5 \text{ W/cm}^2$ ).

One important aspect of the present invention is that the dye (e.g., ICG) can be systemically administered and used with

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great efficacy in the photochemical therapy of diseases characterized by cellular/tissue hyperproliferation and neovascularization; e.g., highly vascularized tumors and their metastases (Kaposi's sarcoma [KS]; adenocarcinoma of the colon, esophagus, breast, etc.; neurofibroma, malignant melanoma), vascular malformations (hemangiomas, port wine stains, varicose veins, telangiectasias) and HPV-induced lesions. In addition, a wide range of hemangiomas, well-vascularized cutaneous metastases, tuberous port wine stains, deep-seated and superficial varicoses and other vascular disorders are treatable using the present method.

The dye may be administered in a concentration range of from 0.5 mg/kg b.w. up to 5 mg/kg b.w., as a bolus or as two or more doses separately administered with an interval of up to 30 min. to saturate the hepatic metabolism of the dye or as a continuous infusion to maintain plasma levels.

ICG can be administered by a variety of routes and/or in a variety of pharmaceutical formulations. A preferred route of administration is parenteral. Examples of parenteral routes of administration include intradermal, intramuscular, intravenous, intraperitoneal, subcutaneous and intranasal routes of administration, preferably intravenous. Suitable pharmaceutical formulations include aqueous solutions, syrups, elixirs,

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tinctures, suspensions with propylene glycol, emulsions and liposomal preparations which prolong the serum half-life of the dye. The preferred formulation is an aqueous solution, and the preferred administration route is i.v. infusion to prolong the serum half-life and result in higher intralesional concentrations and longer circulation times. Based on in vitro studies, however, topical administration may be successful, particularly when dye penetration is enhanced by concurrent application of low frequency ultrasound (e.g., 5-50 kHz, preferably 10-20 kHz), to enhance skin permeation.

For photochemical therapy with ICG, it is possible and desirable to use substantially lower light intensity and lower total light dose than that employed by Chen et al<sup>10</sup> in in vitro photothermal experiments. Successful PDT with elimination of KS skin lesions has been demonstrated by the present inventors using ICG-based light irradiation with a diode laser delivering light at 805 nm (Opto Power Corporation, City of Industry, CA 91745). Most preferably, the total light dose is an order of magnitude lower than that employed for photothermal treatment.

Light irradiation, either continuous or pulsed mode, can be performed directly after intravenous injection, bolus or infusion of dye, at the time of maximal dye concentration in the blood vessels. Alternatively, irradiating can be performed 30 to 60

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min after administration of dye, at a time when the dye has selectively accumulated in the diseased tissue. Selective destruction of the diseased tissue can be achieved either by photothermolysis; that is, either (a) pulsed irradiation matching the thermal relaxation time of blood vessels at the time of maximal dye concentration, or (b) continuous irradiation at the time of exclusive accumulation of the dye in the diseased tissue.

Therapeutic approach:

With the first administration of dye (e.g., bolus injection of ICG, 0.5 mg/kg) the lesion can be diagnosed, indicating permeability, accumulation of the dye and the extent of the lesion (e.g., by determining the difference between ICG-fluorescent tissue and the macroscopically visible area of diseased tissue). At the same time, this first injection will saturate to a certain degree the metabolization capacity of the liver, resulting in a longer serum half-life for a second injection. Thus, the therapeutic window may be extended by additional administrations of dye.

As an alternative, an intravenous continuous infusion or modified dye formulation may yield a longer half-life. However, a short half-life is a definite advantage, because prolonged patient photosensitivity is minimized.

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If necessary or desired, a third administration of dye (e.g., bolus injection of ICG) may complete the coagulation of tissue and vessels. This result is assured since no fluorescence occurs in the successfully treated area. This procedure is typically followed in ophthalmological applications.

ICG, for example, may be administered as an aqueous solution (30-50 ml), either as a bolus or by rapid i.v. infusion. ICG is rapidly removed by the liver from circulating blood (serum half-life = 12 minutes). The dye can be administered rapidly as a single dose, or alternatively, in two or more doses at least 5 minutes (preferably 5-25 minutes) apart.

Solution-based formulations are known in the art, and are prepared by dissolution of the dye and other appropriate additives in the appropriate solvent systems. Such solvents include water, saline, ethanol, ethylene glycol, glycerol, A1 fluid, etc. Suitable additives known in the art include certified dyes, flavors, sweeteners, and antimicrobial preservatives, such as thimerosal (sodium ethylmercurithio-salicylate). Such solutions may be stabilized, for example, by addition of partially hydrolyzed gelatin, sorbitol, or cell culture medium, and may be buffered by methods known in the art, using reagents known in the art, such as sodium hydrogen

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phosphate, sodium dihydrogen phosphate, potassium hydrogen phosphate and/or potassium dihydrogen phosphate.

The preparation of suspensions, for example using a colloid mill, and emulsions, for example using a homogenizer, is known in the art. If the dye (e.g., ICG) is formulated in aqueous propylene glycol, the half life is prolonged, and the irradiation protocol can be modified accordingly.

Parenteral dosage forms, designed for injection into body fluid systems, may require proper isotonicity and pH buffering to the corresponding levels of the patient's body fluids. Parenteral formulations must also be sterilized prior to use.

Isotonicity can be adjusted with sodium chloride and other salts as needed. Other solvents, such as ethanol or propylene glycol, can be used to increase solubility of ingredients of the composition and stability of the solution. Further additives which can be used in the present formulation include dextrose, conventional antioxidants and conventional chelating agents, such as ethylenediamine tetraacetic acid (EDTA).

Directly following the final administration of dye (e.g., a second ICG bolus, 2.5 mg/kg b.w.), light irradiation (e.g., at a power of 2-5 W/cm<sup>2</sup>; dose = 10-250 J/cm<sup>2</sup>, preferably 25-200 J/cm<sup>2</sup>, more preferably about 100 J/cm<sup>2</sup>; wavelength 770-840 nm, preferably about 805 nm) is performed using a continuous or

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pulsed (preferably continuous) diode laser. If ICG accumulates selectively in certain diseased tissues, light irradiation may be performed later, to protect the surrounding normal tissue. For the treatment of larger and deeper vascular malformations, a pulsed diode laser may be necessary. Exact parameters may be determined according to the physician's experiences with a Flashlamp-Pumped-Dye-Laser for smaller and more superficial vascular malformations.

Normally, irradiation is performed only after the final dose, but when the first dose is high (e.g., 2-5 mg/kg b.w.), then irradiation can be performed after each dose of a multiple-dosing regimen. When two or more doses are administered, the first dose is primarily to load the hepatic metabolic system. Other practical procedures can involve commencing the irradiating step during the administering step, in which the dye may be advantageously administered by continuous (preferably slow) infusion.

A typical light dose is 100 J/cm<sup>2</sup>, but the dose can range from 10 J/cm<sup>2</sup> to, for example, 200 J/cm<sup>2</sup>, according to severity of the lesion to be treated. It is highly significant that this light dose is substantially lower than light doses necessary for photothermal effects, which generally require about ten times the present light dose.



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A semi-conductor diode laser may be employed at any wavelength of from 770 to 840 nm, but is advantageously employed at 805 nm.

Treatment of a wide range of medical problems is particularly attractive with dyes such as ICG because the wavelength of light absorption from 770 to 840 nm (preferably at about 800 nm) occurs at wavelengths where competitive absorption from blood and natural body pigments is negligible, thus permitting light penetration to greater depths than at wavelengths less than 770 nm. Interstitial placement of the light source is a practical procedure for larger tumors.

Other features of the present invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention, and are not intended to be limiting thereof.

#### EXAMPLE 1

##### Summary

Indocyanine green (ICG), a dye with an absorption maximum bandwidth of 790 to 810 nm coincident with the emission wavelength of conventional cw-diode laser (805 nm), was investigated *in vitro* using HaCaT keratinocytes (a recently established cell culture model for photodynamic therapy<sup>23</sup>) as a

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model system for concentration-dependent intracellular dye uptake and dye-mediated phototherapy (i.e., cell killing induced by photoactivated ICG). Cellular uptake of ICG, after incubation for 24 hours with ICG concentrations ranging between 1  $\mu\text{M}$  and 50  $\mu\text{M}$ , increased up to an intracellular ICG concentration of  $12.1 \pm 1.3 \text{ nM}/10^6 \text{ cells}$ .

To examine dose dependent effects *in vitro*, keratinocytes were incubated with 0  $\mu\text{M}$  - 50  $\mu\text{M}$  ICG for 24 hours. Subsequently, they were irradiated with laser light of different energy densities (0, 12, 24, 48  $\text{J}/\text{cm}^2$ ) to determine physiotherapeutic efficacy. All applied ICG concentrations above 5  $\mu\text{M}$  led to a cell-killing effect, which depended on ICG concentration and light dose. At 25  $\mu\text{M}$  ICG, cell viabilities for cells kept in the dark (control) and cells treated with 48  $\text{J}/\text{cm}^2$  of 805 nm light were  $0.92 \pm 0.16$  and  $0.12 \pm 0.04$ , respectively.

To study the mechanisms of cell killing, the protective effect of the singlet oxygen quencher sodium azide (rate constant<sup>24</sup>:  $k_q = 5.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) was assessed with regard to the ICG-mediated photokilling of cells. In a quenching experiment, sodium azide (100 mM) was found to be a potent inhibitor of cell killing using 50  $\mu\text{M}$  ICG and 24  $\text{J}/\text{cm}^2$ . Taken together, photoactivation of ICG by 805 nm light was shown to induce cell

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killing of HaCaT keratinocytes, which could be inhibited by the singlet oxygen quencher sodium azide.

Photodynamic treatment with a hematoporphyrin derivative (PHOTOSAN-3) was carried out for comparative purposes. The in vitro data obtained indicate that phototherapy using ICG is a new promising treatment for cancers and/or dermatological conditions.

#### Materials and Methods

Cell culture and dye preparation. The immortalized human keratinocyte cell line HaCaT<sup>25</sup> was maintained in Dulbecco's modified Eagle's medium (Sigma Chemie, Deisenhofen, Germany) supplemented with 5% fetal calf serum (Sigma Chemie) and 1% L-glutamine (Gibco, Eggenstein, Germany) in a humidified atmosphere containing 8% carbon dioxide at 37°C. Cells grown to subconfluence were washed with phosphate buffered saline (PBS; Biochrom, Berlin, Germany) and harvested by a 10 min treatment with 0.1% trypsin/0.04% EDTA (Gibco) in PBS. For in vitro assays, ICG (molar mass of the ICG sodium iodide salt: 924.9 g/mol; PULSION Medizintechnik, München, Germany) and Photosan-3 (Seelab, Wesselburenerkoog, Germany) were dissolved in growth medium at concentrations ranging from 1  $\mu$ M to 50  $\mu$ M.

Cellular uptake of ICG. HaCaT cells ( $1.1 \times 10^6$  cells in 4 ml of growth medium) were inoculated on petri dishes ( $\varnothing$  6 cm;

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Falcon, Becton Dickinson, Heidelberg, Germany). Cells were allowed to attach overnight, and the medium was replaced with 2 ml of ICG solution at each of concentrations of 1, 5, 10, 25 and 50  $\mu\text{M}$ . Cells were incubated for 24 hours at 37°C. The absorption of the supernatants containing ICG was measured at 790 nm using the UV/VIS-Spectrometer Lambda 2 (Perkin-Elmer, Überlingen, Germany). The remaining ICG concentration in the supernatants was calculated by means of a calibration curve which was determined for each experiment in parallel. ICG uptake into the cells was calculated as difference between the initial ICG concentration and the ICG concentration of the supernatant removed from the cells after 24 hours.

Treatment protocol and proliferation assay. HaCaT cells were seeded at equal concentrations ( $15 \times 10^3$  cells in 100  $\mu\text{l}$  medium per well) into 96-well microtitre plates (Costar, Tecnomara, Fernwald, Germany). After cell attachment overnight, medium was replaced with 100  $\mu\text{l}$  of an ICG solution having a concentration of 5, 10, 25 or 50  $\mu\text{M}$ . Following incubation for 24 hours at 37 °C, supernatants were removed, cells were carefully washed with medium to eliminate any remaining dye then covered with 100  $\mu\text{l}$  of dye-free and drug-free medium immediately before irradiation. Control cells were processed identically, except

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that dye (ICG or PHOTOSAN-3) was not present in the solution replacing the medium used for cell attachment.

ICG incubated cells were irradiated using a cs-diode laser at 805 nm (Opto Power Corp., City of Industry, CA 91745) with 15 W maximum optical output power. Laser light was coupled into a moncore fiber having a 1500  $\mu\text{m}$  diameter and was distributed by a biconvex lens to a flat homogeneous area ( $150\text{ cm}^2$ ,  $\varnothing\ 14\text{ cm}$ ) sufficient to cover a 96-well microtitre plate. The fluence rate to which cells were exposed was adjusted to  $40\text{ mW/cm}^2$ . Three different total light doses were used: 12, 24 and  $48\text{ J/cm}^2$ . Temperature measurements of light-treated media ensured that no hyperthermic conditions were imposed by this irradiation arrangement.

Following irradiation, cells were maintained under normal culture conditions for 24 hours. Proliferation of cells after ICG and/or light treatment was assessed by means of the 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay<sup>26,27,28</sup>. Briefly, 10  $\mu\text{l}$  of MTT solution (5 mg/ml in PBS; Sigma Chemie) was added to each well containing 100  $\mu\text{l}$  of growth medium. After 4 hours of incubation, 100  $\mu\text{l}$  of sodium dodecyl sulphate solution (20% in aqua dest; Merck, Darmstadt, Germany) was added to each well. The plates were left overnight at  $37^\circ\text{C}$ , and the absorption of the dissolved metabolic product formazan

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was measured at 540 nm using an Emax microplate reader (Molecular Devices, Menlo Park, CA). The cell viability (CV) was determined as the ratio of the optical density of the treated cells to the optical density of the untreated control cells.

Effect of sodium azide on cell killing. To study the mechanisms of cell killing by photoactivation of ICG, sodium azide, an effective physical quencher of singlet oxygen<sup>29,30,31</sup>, was added to the cell culture prior to irradiation. HaCaT cells treated with 50  $\mu$ M ICG for 24 hours were irradiated as described above (24 J/cm<sup>2</sup>) but in presence of sodium azide (Merck) at concentrations of 10, 50 or 100 mM each in PBS, selected to provide optimum protection and tolerable dark toxicity. Cell viability (CV) was assessed using the MTT assay described above. The quenching effect, expressed as the percentage of protection, was determined as the ratio of the CV of cells treated with ICG and light in presence of the quencher to the CV of cells treated with ICG and quencher but without light.

As a positive control, each experiment was repeated using the hematoporphyrin derivative PHOTOSAN-3. The role of singlet oxygen as the predominant oxidizing agent in photodynamic therapy (type II reaction) has been reported by numerous investigators (for a review, see: Henderson and Dougherty, 1992<sup>32</sup> and Pass, 1993<sup>33</sup>). Cells incubated with 2.5  $\mu$ g/ml PHOTOSAN-3 for 24 hours

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were irradiated with a lamp emitting incoherent light<sup>23</sup> (PDT 1200, Waldmann Medizintechnik, VS-Schwenningen, Germany). The fluence rate and light dose to which cells were exposed were adjusted to 40 mW/cm<sup>2</sup> and 24 J/cm<sup>2</sup>, respectively.

Data analysis and statistics. Each individual experiment was carried out at least in triplicate. For the proliferation assay, at least 10 individual wells were plated with cells treated in an identical matter, and their mean optical density was used for data analysis. The effects of the different treatment modalities were characterized as CV of treated cells compared with non-irradiated controls. Differences were tested for statistical significance using the two-sided t-test. All primary data are presented as a means with standard deviations of the mean.

## Results and Discussion

### ICG uptake in vitro

Quantitation of dye uptake into keratinocytes after 24 hours of incubation with different ICG concentrations ranging from 1  $\mu$ M to 50  $\mu$ M showed that the intracellular ICG concentration increased significantly with the extracellular dye concentration ( $n = 6$ ,  $p < 0.05$ ; see Fig. 1). The intracellular ICG concentration was maximal at  $12.1 \pm 1.3$  nM/10<sup>6</sup> cells (i.e., (7.3

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$\pm 0.8) \times 10^9$  molecules/cell) when a concentration of 50  $\mu\text{M}$  was used. Assuming an average cell volume, intra/extracellular concentration ratios were estimated at 230 and 130 for 1  $\mu\text{M}$  and 50  $\mu\text{M}$  ICG, respectively. The relation between intracellular uptake and ICG concentration was characterized by Michaelis-Menten kinetics:  $V_{\text{max}} = 25.6 \mu\text{g}$  and  $K_m = 47.0 \mu\text{M}$ . This non-linear uptake pattern together with the high intra/extracellular concentration ratios presumably indicate a non-diffusive mechanism of cellular drug uptake.

#### ICG phototoxicity in vitro

Proliferation assays 24 hours after irradiation exhibited a concentration dependent CV as illustrated in Fig. 2. Irradiation alone did not lead to a significant decrease in CV. Increasing ICG concentration led to a maximum dark toxicity of 15% (CV =  $0.85 \pm 0.16$ ) at the highest concentration (statistically significant for 25  $\mu\text{M}$  and 50  $\mu\text{M}$  ICG;  $n = 4$ ,  $p < 0.05$ ). Incubation with ICG and light treatment reduced the CV significantly at all ICG concentrations above 5  $\mu\text{M}$  ( $n = 3$ ,  $p < 0.001$ ) (Fig. 2). With regard to the dark toxicity, an ICG concentration of 25  $\mu\text{M}$  and a light dose of 48 J/cm<sup>2</sup> seemed to be the optimal treatment modality in this study (CV =  $0.12 \pm 0.04$ ; dark toxicity 8%).



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Light dose finding studies (12 J/cm<sup>2</sup>, 24 J/cm<sup>2</sup>, 48 J/cm<sup>2</sup>) showed that the CV significantly decreased with increasing light dose (n=3, p<0.01 except for 5 μM). Microscopic investigation of the cells 24 hours after treatment with an ICG concentration of 50 μM and a light dose of 48 J/cm<sup>2</sup> (CV = 0.07 ± 0.02) revealed that the remaining cell fraction was heavily damaged. Cells were rounded up, aggregated and only few showed mitochondrial MTT staining. Combining a 24 hour ICG incubation with subsequent 805 nm laser light irradiation significantly reduced the viability of HaCaT cells.

#### Effect of sodium azide on cell killing

Sodium azide significantly inhibited the ICG-mediated photokilling (50 μM ICG, 24 J/cm<sup>2</sup>) of HaCaT keratinocytes. As shown in Table 1, the protective effect depended on the sodium azide concentration. Cell viabilities after quenching with sodium azide significantly differed from CV without quenching (n=3, p<0.05). The most pronounced effect was achieved for a concentration of 100 mM sodium azide, resulting in 97 ± 8% protection. A comparable effect was found for photodynamic treatment with Photosan-3 at concentration of 2.5 μg/ml (CV = 0.05 ± 0.04) in presence of 100 mM sodium azide. Sodium azide is

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a well-known quencher of singlet oxygen and is also known to react with hydroxyl radicals and other radical species<sup>34</sup>.

Table 1. Effect of sodium azide on drug-mediated photokilling of HaCaT keratinocytes (incubation time 24 hours; total light dose 24 J/cm<sup>2</sup>)

Sodium azide concentration	% Protection	
	ICG (50 $\mu$ M) + cw-diode laser	Photosan-3 (2.5 $\mu$ g/ml) + PDT 1200
10 mM	46 $\pm$ 9	18 $\pm$ 3
50 mM	86 $\pm$ 2	63 $\pm$ 5
100 mM	97 $\pm$ 8	95 $\pm$ 14

The effectiveness of sodium azide in this in vitro system was demonstrated using the established photodynamic treatment with PHOTOSAN-3. Photodynamic therapy with this hematoporphyrin derivative, the current sensitizer of choice, is known to generate a number of excited oxygen species, mainly singlet oxygen, which are responsible for cell death<sup>35,36,37,38,39</sup>. The results of this experiment suggest that photoactivation of ICG in vitro generates radical species which mediate cell death and which can be scavenged by sodium azide during light treatment, resulting in an increase of CV.

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A destructive thermal effect has been exploited in vivo for ICG-enhanced photocoagulation<sup>19,20</sup> or tissue welding<sup>21,22</sup>. However, the photothermal injury observed in these in vivo models was obtained at much higher laser power densities of 1 to 20 W/cm<sup>2</sup>. In the present experimental setup using low fluence rates of 40 mW/cm<sup>2</sup>, no rise of temperature could be measured after irradiation. In addition, cell killing could be inhibited by sodium azide. Therefore we may conclude that photodynamic effects dominate over photothermal effects in the present ICG-mediated photokilling of HaCaT keratinocytes. The results support a photodynamic mechanism for light-induced cell killing mediated by ICG.

#### Conclusion

The purpose of Example 1 was to examine the cellular uptake of ICG and to evaluate the effectiveness of ICG-mediated phototherapy in vitro. The results reveal that the viability of HaCaT cells, which take up ICG in an accumulative manner, was significantly reduced by photoactivation of ICG, depending to some extent on chromophore concentration and light dose. Cell killing induced by administering the highest ICG concentration (50  $\mu$ M) and irradiating with 805 nm light could be inhibited by sodium azide. This inhibition suggests involvement of reactive

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(e.g., singlet) oxygen species. Because of its non-toxicity in vivo and its high absorption cross-section ( $\sigma = 8 \times 10^{-16} \text{ cm}^2$  in water<sup>15</sup>) in the therapeutic window of light (600 - 1200 nm), dyes having the physicochemical properties of ICG are new and useful candidates as sensitizers for photodynamic therapy.

#### EXAMPLE 2

Example 2 is a case report demonstrating the effectiveness of photodynamic therapy (PDT) with indocyanine green (ICG) for AIDS-related Kaposi's sarcoma (KS). Various KS lesions of a 32-year old male homosexual patient having AIDS-related KS since 1994, diagnosed with AIDS on 10/94, and having no other opportunistic infections were treated with ICG-PDT. One superficial KS lesion (1.2 x 0.5 cm) was located on the patient's upper left back.

#### Treatment Protocol with ICG-PDT:

03/21/95: Bolus injection of ICG (2.5 mg/kg b.w.); after 30 min, second bolus injection of ICG (2.5 mg/kg b.w.); 1 min after second bolus, irradiation of KS lesion with a cw diode laser (805 nm, intensity 3 W/cm<sup>2</sup>, exposure time 33 seconds; total light dose 100 J/cm<sup>2</sup>).

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03/21/95: whitish discoloration with reflex erythema immediately after irradiation

03/22/95: development of blister, sharply demarcated to the former lesion

03/24/95: superficial erosion

03/29/95: superficial erosion

07/14/95: complete healing, slight hyperpigmentation

08/16/95: complete healing, only discrete atrophic scar, no clinical sign of tumor residue

Histology (taken from another KS site at the forearm):

03/21/95: KS lesion before therapy, H & E stain

03/22/95: coagulation, fibrinoid necrosis and homogenization of epidermis and dermis, H & E stain

03/22/95: denudation of small venous vessel on one side in a depth of 3.5 mm, H & E stain

Conclusion:

This case report demonstrates that AIDS-related Kaposi's sarcoma can be treated by phototherapy using ICG safely and effectively.

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Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

References:

The following references are incorporated herein by reference in their entireties.

1. Fox, I. J., L. G. S. Brooker, D. W. Heseltine, H. E. Essex and E. H. Wood (1956) New dyes for continuous recording of dilution curves in whole blood independent of variations in blood oxygen saturation. (Abstr.) Am. J. Physiol. 187, 599.
2. Fox, I. J. and E. H. Wood (1960) Indocyanine green: physical and physiologic properties. Mayo Clin. Proc. 35, 732-744.
3. Bradley, E. C. and J. W. Barr (1968) Determination of blood volume using indocyanine green dye. Life Sci. 7, 1001-1007.
4. Leevy, C. M., F. Smith, J. Longueville, G. Paumgartner and M. Howard (1967) Indocyanine green clearance as a test for hepatic function. Evaluation by dichromatic ear densitometry. Jama 200, 236-240.
5. Flower, R. W. and B. F. Hochheimer (1976) Indocyanine green dye fluorescence and infrared absorption choroidal angiography performed simultaneously with fluorescein angiography. John Hopkins Med. J. 138, 33-42.
6. Moneta, G., M. Brülisauer, K. Jäger and a. Bollinger (1987) Infrared fluorescence videomicroscopy of skin capillaries with indocyanine green. Int. J. Microcirc. Clin. Exp. 6, 25-34.

-29-

7. Hammond, D. C., F. R. Lane, R. A. Welk, M. J. Madura, D. K. Borreson and W. J. Passinault (1988) Endoscopic tattooing of the colon. An experimental study. Am. Surg. 55, 457-461.
8. Chance, B., K. Kang, L. He, J. Weng and E. Sevick (1993) Highly sensitive object location in tissue models with linear in-phase and anti-phase multi-element optical arrays in one and two dimensions. Proc. Natl. Acad. Sci. USA 90, 3423-3427.
9. Benya, R., J. Quintana and B. Brundage (1989) Adverse reactions to indocyanine green; a case report and a review of the literature. Cathet. Cardio. Diagnosis 17, 231-233.
10. Hope-Ross, M., L. A. Yannuzzi, E. S. Gragoudas, D. R. Guyer, J. S. Slakter, J. A. Sorenson, S. Krupsky, D. A. Orlock and C. A. Puliafito (1994) Adverse reactions due to indocyanine green. Ophthalmology 101, 529-533.
11. Gathje, J., R. R. Steuer and K. R. K. Nicholes (1970) Stability studies on indocyanine green dye. J. Appl. Physiol. 29, 181-185.
12. Simmons, R. and R. J. Shephard (1971) Does indocyanine green obey Beer's law? J. Appl. Physiol. 30, 502-507.
13. Tripp, M. R., G. M. Cohen, D. A. Gerasch and I. J. Fox (1973) Effect of protein and electrolyte on the spectral stabilization of concentrated solutions of indocyanine green. Proc. Soc. Exp. Biol. Med. 143, 879-883.
14. Landsman, M. L. J., G. Kwant, G. A. Mook and W. G. Zijlstra (1976) Light-absorbing properties, stability, and spectral stabilization of indocyanine green. J. Appl. Physiol. 40, 575-583.
15. Philip, R., A. Penzkofer, W. Bäumlner, R. M. Szeimies and M. Landthaler (1995) Absorption and fluorescence spectroscopic investigation of indocyanine green. To be submitted.
16. Paumgartner, G., P. Probst, R. Kraines and C. M. Leevy (1970) Kinetics of indocyanine green removal from the blood. NY Acad. Sci. 170, 134-170.
17. Thiessen, J. T., P. L. Rappaport and J. G. Eppel (1984) Indocyanine green pharmacokinetics in the rabbit. Can. J. Physiol. Pharmacol. 62, 1078-1085.

-30-

18. DeCoste, S. D., W. Farinelli, T. Flotte and R. R. Anderson (1992) Dye-enhanced laser welding for skin closure. Laser Surg. Med. 12, 25-32.
19. Kirsch, A. J., G. E. Dean, M. C. Oz, S. K. Libutti, M. R. Treat, R. Nowygrod and T. W. Hensle (1994) Preliminary results of laser tissue welding in extravesical reimplantation of the ureters. J. Urol. 151, 514-517.
20. Libutti, S. K., M. C. Oz, R. S. Chuck, J. S. Auteri, M. R. Treat and R. Nowygrod (1991) A preliminary study of dye-enhanced laser photosclerosis. Surgery 109, 163-168.
21. Chong, L. P., S. A. Özler, J. D. de Queiroz and P. E. Liggett (1993) Indocyanine green-enhanced diode laser treatment of melanoma in a rabbit model. Retina 13, 251-259.
22. Reichel, E., C. A. Puliafito, J. S. Duker and D. R. Guyer (1994) Indocyanine green dye-enhanced diode laser photocoagulation of poorly defined subfoveal choroidal neovascularization. Ophthalm. Surg. 25, 195-201.
23. Szeimies, R. M., R. Hein, W. Bäuml, A. Heine and M. Landthaler (1994) A possible new incoherent lamp for photodynamic treatment of superficial skin lesions. Acta Derm. Venereol. (Stockh) 74, 117-119.
24. Hall, R. D. and C. F. Chignell (1987) Steady-state near-infrared detection of singlet molecular oxygen; a Stern-Volmer quenching experiment with sodium azide. Photochem. Photobiol. 45, 459-464.
25. Boukamp, P., R. T. Petrussevska, D. Breitkrutz, J. Hornung, A. Markham and N. E. Fusenig (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell. Biol. 106, 761-771.
26. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55-63.
27. Niks, M. and M. Otto (1990) Towards an optimized MTT assay. J. Immunol. Methods 130, 149-151.



-31-

28. McHale, A. P. and L. McHale (1988) Use of a tetrazolium based colorimetric assay in assessing photoradiation therapy in vitro. Cancer Lett. 41, 315-321.
29. Ito, T. (1978) Cellular and subcellular mechanisms of photodynamic action: the  $^1O_2$  hypothesis as a driving force in recent research. Photochem. Photobiol. 28, 493-508.
30. Agawal, R., M. Athar, D. R. Bickers and H. Mukhtar (1990) Evidence for the involvement of singlet oxygen in the photodestruction by chloroaluminium phthalocyanine tetrasulfonate, Biochem. Biophys. Res. Comm. 173, 34-41.
31. Hampton, J. A., D. Skalkos, P. M. Taylor and S. H. Selman (1993) Iminium salt of copper benzochlorin (CDSI), a novel photosensitizer for photodynamic therapy: mechanism of cell killing. Photochem. Photobiol. 58, 100-105.
32. Henderson, B. W. and T. J. Dougherty (1992) How does photodynamic therapy work? Photochem. Photobiol. 55, 145-157.
33. Pass, H. I. (1993) Photodynamic therapy in oncology: mechanism and clinical use. J. Natl. Cancer Inst. 85, 443-456.
34. Singh, A., G. W. Koroll and R. B. Cundall (1981) Azide radical formation from sodium azide and hydroxyl and its reactions with tryptophan and tyrosine. In Oxygen and Oxy-radicals in Chemistry and Biology (Edited by M. A. J. Rodgers and E. L. Powers), Pp. 739-741. Academic Press, New York.
35. Weishaupt, K. R., C. J. Gomer and T. J. Dougherty (1976) Identification of singlet oxygen as the cytotoxic agent in photo-inactivation of a murine tumor. Cancer Res. 36, 2326-2329.
36. Moan, J., E. O. Pettersen and T. Christensen (1979) The mechanism of photodynamic inactivation of human cells in vitro in the presence of hematoporphyrin. Br. J. Cancer 39, 398-407.
37. Henderson, B. W. and A. C. Miller (1986) Effects of scavenger of reactive oxygen and radical species on cell survival following photodynamic therapy in vitro: comparison to ionizing radiation. Radiation Res. 108, 196-205.
38. Keene, J.P., D. Kessel, E. J. Land, R. W. Redmond and T. G. Truscott (1986) Direct detectio of singlet oxygen sensitized by

-32-

hematoporphyrin and related compounds. Photochem. Photobio. 43, 117-120.

39. Gomer, C. J., A. Ferrario, N. Hayashi, N. Rucker, B. C. Szirth and A. L. Murphree (1988) Molecular, cellular, and tissue responses following photodynamic therapy. Laser Surg. Med. 8, 450-463.

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## CLAIMS:

1. A method of treating cancer, a dermatological disease or condition, or combination thereof, comprising the steps of:  
administering to a patient in need of such treatment an effective amount of indocyanine green, and  
irradiating the affected or apparently affected tissue of the patient with a dose of light having a wavelength within the range of from 770 to 840 nm, the dose of light being effective to therapeutically treat the cancer, dermatological disease or condition, or combination thereof.
2. The method of Claim 1, wherein said dose of light is ineffective to thermally destroy the irradiated tissue.
3. The method of Claim 1, wherein said dose of light is 200 J or less.
4. The method of Claim 3, wherein said dose of light is from 25 J to 200 J.
5. The method of Claim 4, wherein said dose of light is from 50 J to 150 J.

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6. The method of Claim 1, wherein said cancer, dermatological disease or condition, or combination thereof, is a cancer.

7. The method of Claim 6, wherein said cancer is Kaposi's sarcoma.

8. The method of Claim 1, wherein said light has a wavelength in the range of from 790 to 810 nm.

9. The method of Claim 8, wherein said light has a wavelength of about 805 nm.

10. The method of Claim 1, wherein said administering step comprises administering a first dose of indocyanine green, waiting for a period of time of at least 5 minutes, then administering a second dose of indocyanine green.

11. The method of Claim 1, wherein said irradiating step is commenced during the administering step.

12. The method of Claim 1, wherein said irradiating step is conducted at a power density of from 5 mW/cm<sup>2</sup> to 5 W/cm<sup>2</sup>.

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13. The method of Claim 12, wherein said irradiating step is conducted at a power density of from 10 mW/cm<sup>2</sup> to 3 W/cm<sup>2</sup>.

14. The method of Claim 13, wherein said irradiating step is conducted at a power density of from 25 mW/cm<sup>2</sup> to 2 W/cm<sup>2</sup>.

15. The method of Claim 1, wherein the light is generated by a diode laser.

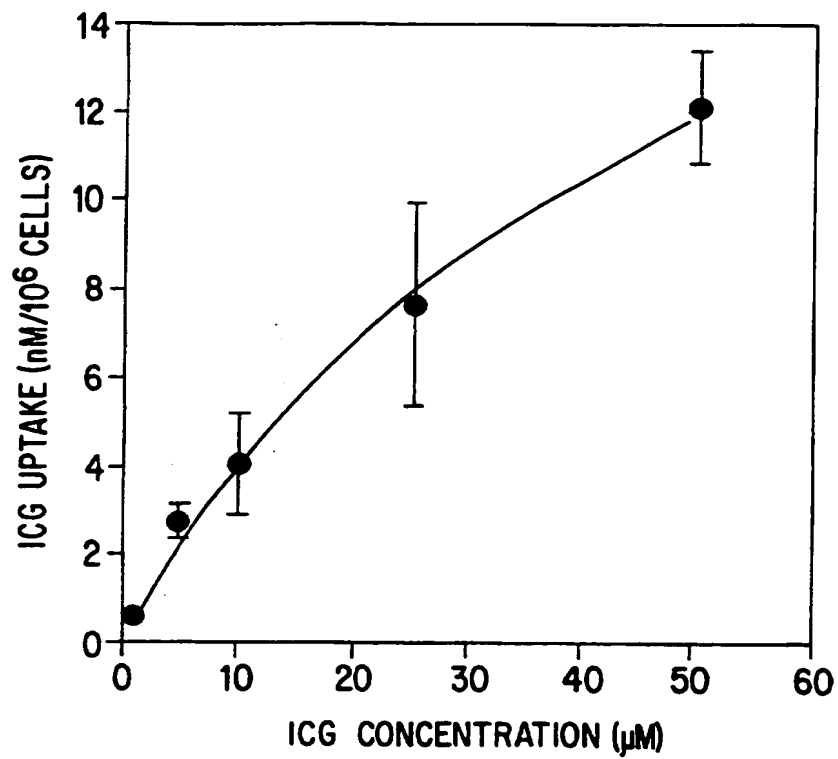


FIG. 1

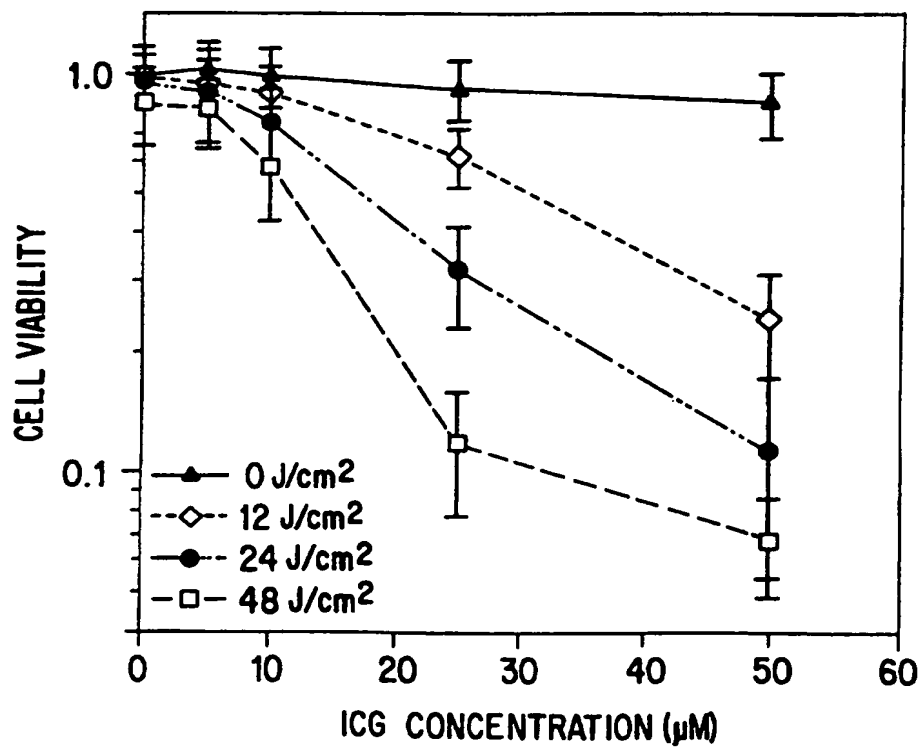


FIG. 2

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/01927

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61B 19/00; A61M 31/00

US CL : 128/898; 604/49

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 128/898; 604/49; 606/3; 607/901

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS, DIALOG

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEN et al. Chromophore-enhanced laser-tumor tissue photothermal interaction using an 808-nm diode laser. Cancer letters. 1995, Vol.88, No.1, pages 15-19, see entire document.	1-15
X	Chen et al. Chromophore-enhanced in vivo tumor cell destruction using an 808-nm diode laser. Cancer letters. 1995, Vol.94, No.2, pages 125-131, see entire document.	1-15
X	CHEN et al. Photothermal effects on murine mammary tumors using indocyanine green and an 808-nm diode laser: an in vivo efficacy study. 1996, Vol.98, No.2, pages 169-173, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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